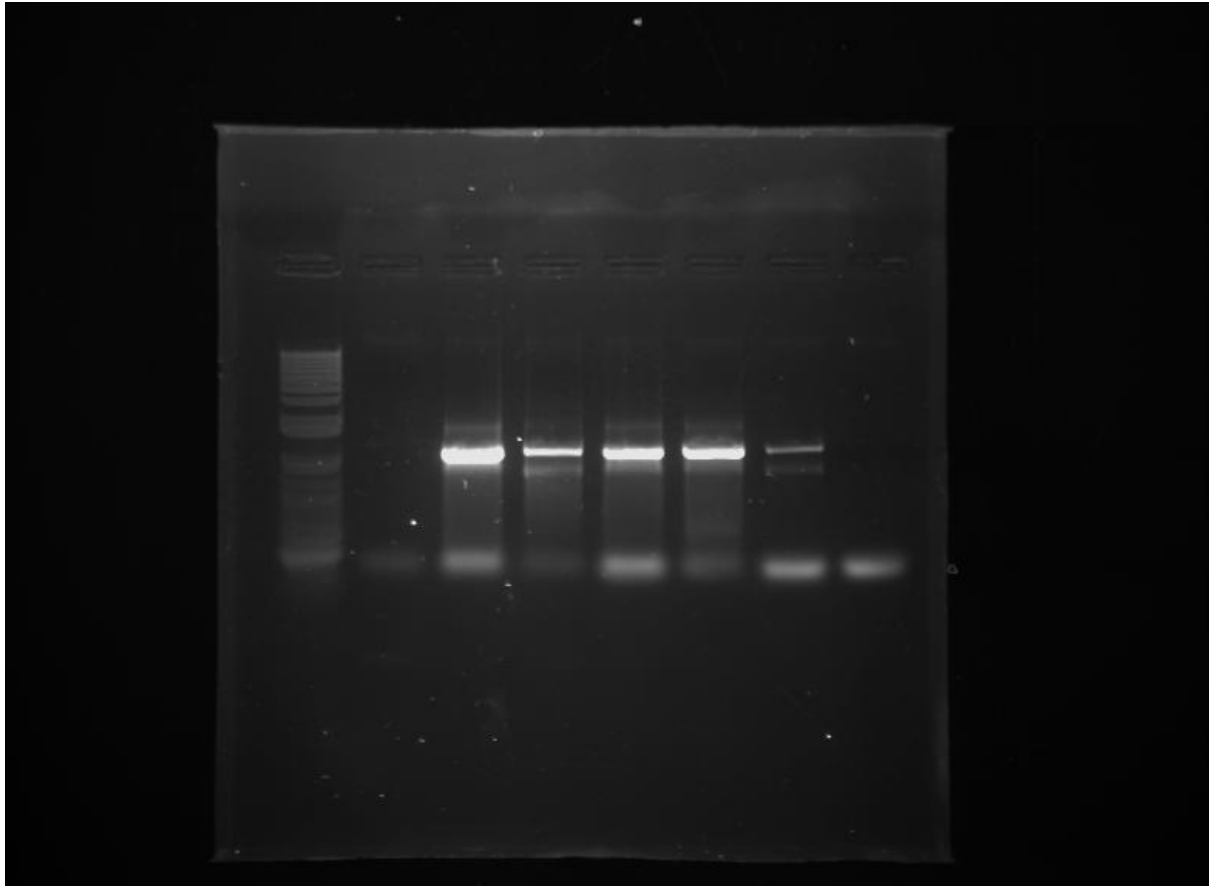


**12<sup>th</sup> October**

**Gel electrophoresis of PCR products of pBAV1K miniprepmed from KI on 11<sup>th</sup> October**

- 1% agarose gel, 100V, 20 minutes
- Ladder: NEB 2-Log ladder?
- Expected size: 984bp
- If NEB 2-log ladder, positives are: pBAV1K numbers 2-5, 6- Pos control, 7- Neg. control.



**Review of ATCC 53582 and KI feedstocks experiment from 3<sup>rd</sup> September**

- In feedstocks experiment, KI produces a visibly smaller pellicle, but almost all KI cultures are filled with a thick gelatinous substance, much like ATCC cultures. However, in cultures 0903MFVa-d, which were seeded for KI gDNA extraction from samples 0815MFVa, there is no gelatinous substance present. The difference could be due to the fact that in feedstocks experiment, tube caps were opened several times in order to allow increased diffusion of air into the culture medium, whereas the gDNA tubes were kept closed. However, it could also be due to ATCC strain contamination of KI strain cultures, producing similarly gelatinous substance in the KI feedstocks experiment cultures.
- KI gDNA cultures also contain visible amount of gelatinous substance, attached to the pellicle, but much less than that present in KI feedstocks experiment cultures. This indicates that the production of gelatinous substance is not only characteristic of ATCC strain. Furthermore, all negative controls were clear, indicating that this was not contamination KI cultures by ATCC strain.

- Cultures grown in 20ml in 50ml tubes standing

## **11<sup>th</sup> October**

### **Plating transformed KI and ATCC 53582 cells**

- Prepared HS-agar –chloramphenicol plates as follows:
  - For KI:
    - 6x Cam, 8x Cam, 10x Cam
  - For ATCC
    - 3x Cam, 6x Cam, 1x Cam-cellulase, 4x Cam-cellulase
- Plated each of KI 800ul overnight culture in 3 aliquots onto 6x, 8x, 10x Cam plates,
- Plated each of ATCC 53582 800ul overnight culture onto 4 of each of 3xCam, 6xCam, 1xCam-Cellulase, 4x Cam-Cellulase plates.
- Due to lack of time, prepared 1xCam-cellulase and 4xCam-Cellulase HS-Agar using microwave oven – there was some evaporation of water, so final solution was likely more concentrated than original HS-medium. Added 0.1% cellulase and chloramphenicol to liquid HS-agar after it had cooled to 50degC.
- Plated out 100ul of each culture onto respective plates

### **PCR of miniprep pBAV1k from transformed KI**

- Used GoTaq master mix, 20ul reaction volume, primers iGEM55, iGEM56, 1ul DNA from each of miniprep pBAV1K DNA.

- 

## **10<sup>th</sup> October**

### **Transformation of electrocompetent KI and ATCC 53582 with verified biobrick-pSEVA331 and pSEVA321 constructs using electroporation**

- Used 50ul aliquots of cells for electroporation
- Used 2ul of DNA, 1ul if arced
- Resuspended electroporated cells in 800ul HS-cellulase media, kept on room temp until all electroporations finished, then placed on 30C, 180rpm shaking incubator overnight

## **9<sup>th</sup> October**

## **6<sup>th</sup> October**

## **5<sup>th</sup> October**

### **Culture PCR of cultures inoculated on 4<sup>th</sup> October**

- Ran a culture PCR of 50 cultures:

- For a 20µl reaction volume: 20ul 250
- GoTaq® Green Master Mix, 2X 10µl 125
- Upstream primer, iGEM53, 10µM 1ul 125
- Downstream primer iGEM54, 10µM 1ul 12.5
- DNA template colony aliquot
- Nuclease-Free Water 8ul 100ul
- Thermocycler conditions same as 4<sup>th</sup> Oct.

#### Plans:

#### 4<sup>th</sup> October

##### Transformation of biobricks into *G.xylinus* using electroporation

- Same conditions as before, except 33ul of competent cells used, 2ul of DNA added

#### 3<sup>rd</sup> October

##### Culture PCR of pSEVA321-bb and pSEVA331-bb ligated with various parts

- For a 20µl reaction volume: 20ul 500ul
- GoTaq® Green Master Mix, 2X 10µl 250
- Upstream primer, iGEM53, 10µM 1ul 25
- Downstream primer iGEM54, 10µM 1ul 25
- DNA template colony 0.2
- Nuclease-Free Water 8ul 200

##### Analyze colony PCR results using agarose gel electrophoresis

##### In total 120 samples

- Amplicon size without insert: 220bp
- Amplicon size with insert: variable, up to 2kbp
- Used a 1.5% agarose gel for electrophoresis
- Total number of samples: 120
- Total number of gels (if two rows are used) 9

##### Miniprepping out positive cultures

- 

##### Colony PCR of growing cultures to determine the cultures containing correct plasmids

-

### **Transformation of biobricks subcloned into pSEVA-BB results**

- Colonies have been grown for 3 days – many show light pink coloration, indicating that the plasmid copy number is low, resulting in low expression levels
- Two plasmids were used: pSEVA321 and pSEVA331. Most are only lightly coloured, however HM2 shows high level of expression. However, HM2 was ligated into pSEVA331 as most other constructs, so copy number there can not be the only variable.
- 

**2<sup>nd</sup> October**

### **Inoculations of LB-chloramphenicol media with colonies transformed in 1<sup>st</sup> October**

#### **Colony PCR using Green GoTaq for screening of correct inserts**

- Prepared a master mix using 2X Green GoTaq mastermix,
- For a 20µl reaction volume:                      20ul                      1050ul
- GoTaq® Green Master Mix, 2X                      10µl                      525
- upstream primer, 10µM                      1ul                      52.5
- downstream primer, 10µM                      1ul                      52.5
- DNA template                      colony                      -
- Nuclease-Free Water                      8ul                      420
- 
- Number of reactions:  $34 \times 6 = 204$  reactions in total -> 4080 ul of the total master mix. 4x 1050ul in 1.5ml tubes
- Performed the colony PCR by first rubbing colonies into PCR tubes, then adding the total prepared master mix
- Thermocycler conditions: touchdown PCR with 15 cycles touchdown and 30 cycles amplification
- 

**1<sup>st</sup> October**

### **Ligation of restricted Biobricks into pSEVA321-BB and pSEVA331-BB backbones**

- Ligation products transformed into DH10B and Dh5alpha chemically competent cells

### **Measuring the ATCC 53582 gDNA library quality and fragment size distribution using Bioanalyzer 2100**

- Used the DNA high sensitivity chip
- Performed a 2x serial dilution, added each dilution to the chip twice
- Ran the bioanalyzer according to manufacturer's instructions

**30<sup>th</sup> September**

**Preparation of a second ATCC 53582 gDNA library for genome sequencing using Nextera kit**

- 

**29<sup>th</sup> September**

**Measuring the ATCC53582 and KI-G.xylinus gDNA library range using Bioanalyzer 2100**

- Performed a 2xdilution series, with 2x – 32x diluted library,
- Used the high sensitivity chip according to manufacturer's instructions
- 

**28<sup>th</sup> September**

**PCR of various parts for conversion into BB format.**

- **Amplifying out RBS-sfGFP-terminator construct for insertion behind arabinose inducible operon**
  - Forward primer: iGEM97(Tm 70, 52), reverse primer iGEM80 (tm 58)
  - DNA: XSM plasmid 13 (Bba\_I20260)
- **Putting Vhb coding sequence into BB format:**
  - Forward primer: iGEM102,(Tm 69,52) Reverse primer iGEM103(Tm 70, 49)
  - DNA:pBla-Vhb-122
- **Putting Vhb CDS behind Anderson promoters:**
  - Forward primer:iGEM100 (Tm:66, 52), reverse primer iGEM101 (Tm: 66, 49)
  - DNA: pBla-Vhb-122

PCR reaction conditions:

- Q5 polymerase, using touchdown 15 cycles, elongation 30 cycles, annealing temp. 52C
- Use both GC and HF buffer for both, 2x GC replicates for each
- PCR extraction of BioBricks no 28- Forward primer iGEM79(56), iGEM80(58)
- Use Q5 with both HF and GC buffer for each biobrick
- Annealing temp 50C for all

**Q5 master mix same for both**

- Using Q5 polymerase, HF buffer

| Component                       | 25 µl Reaction | Final Concentration |
|---------------------------------|----------------|---------------------|
| 5X Q5 Reaction Buffer           | 5 µl           | 1X                  |
| 10 mM dNTPs                     | 0.5 µl         | 200 µM              |
| 10 µM Forward Primer            | 1.25 µl        | 0.5 µM              |
| 10 µM Reverse Primer            | 1.25 µl        | 0.5 µM              |
| Template DNA                    | 0.5ul          | < 1,000 ng          |
| Q5 High-Fidelity DNA Polymerase | 0.25 µl        | 0.02 U/µl           |

| Component           | 25 µl Reaction | Final Concentration |
|---------------------|----------------|---------------------|
| Nuclease-Free Water | 16.25          |                     |

- Part 28 – primers

### Subcloning of various parts into biobricked pSEVA321 and pSEVA331

- DpnI treated PCR parts overnight, then heat inactivated at 80C, 20min.
- PCR purified using Qiagen PCR purification kit – purified it twice, as there was guanidinium HCl contamination present; lost all of the DNA, indicating malfunctioning of the kit.
  - Recommendations: use MinElute columns and wash twice with PE buffer to remove Guanidinium HCl
- Double digest PCR parts:
  - Cutting with XbaI, PstI:
    - Vhb-BB (use full volume)+ -> PCR purify
    - 13 (BBa\_I20260) (use full volume)+ -> PCR purify->Dephosphorylate->ligate
    - 28 (BBa\_J23112) (use full volume)+ ->PCR purify
      - 5ul of Cutsmart Buffer, 0.5ul of each enzyme, 16ul of H2O
    - pSEVA321-BB+ (50ul reaction)->PCR purify
    - pSEVA331-BB+ (50ul reaction) -> PCR purify
    - pSEVA351-BB (50ul reaction, 1ug of DNA) -> PCR purify
      - use 5ul of CutSmart buffer, 0.5ul of each enzyme
    - All CBDs+ -> gel purify (20ul RE reaction)
      - 6ul of DNA, 2ul of Cutsmart, 0.5ul of each enzyme, 11ul of H2O
    - 7E,2 + -> gel purify (20 ul RE reaction)
      - Use 1ug -2ug of DNA, 2ul of CutSmart buffer, 0.5ul of each enzyme, water as needed
  - Cutting with XbaI, SpeI
    - 7E,2 (Bba\_K808000) (followed by gel purification and 3 part ligation of ligation with 13 PCR product and pSEVA backbones)
      - Use 1-2ug of DNA, in 20ul reaction, 0.5ul of each enzyme, water as needed
- Gel purification and checking:
  - Purification of all CBDs
  - Check reaction of 7E,2 pSEVA321-BB and pSEVA331-BBs
    - CBD-sfGFP sizes: 700bp+
    - 7E2 size:
    - pSEVA321 and pSEVA331 sizes (also add unrestricted controls):

27<sup>th</sup> September

Antibiotic concentrations on plates required for culturing only transformed KI and ATCC cells, without any background colonies:

- Kombucha-isolated acetobacter:

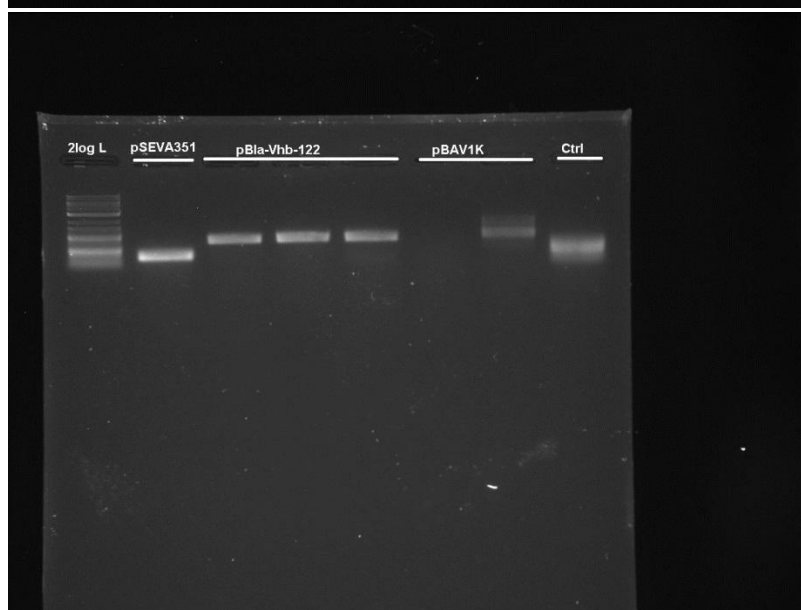
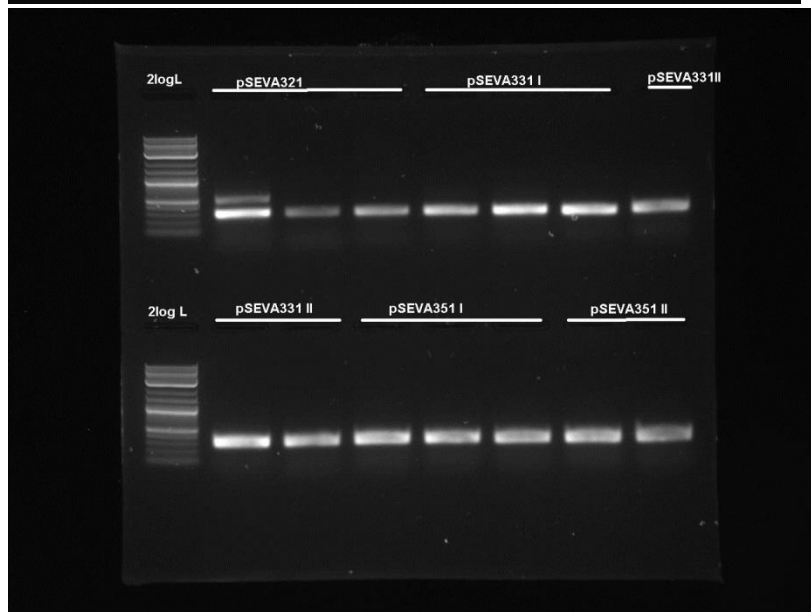
- Chloramphenicol: no colonies present at 7x, 10x chloramphenicol, colonies present at 5x. Therefore, need to grow at least 7x Cam concentration
- Ampicillin: no colonies present on 5x, 7x, 10x ampicillin concentrations, thus need to grow them at least at 5x concentration
- Kanamycin: colonies present at 5x, 7x kanamycin concentration; no colonies present at 10x concentration. Therefore need to grow at least at 10x concentration. NB! pBla and pBAV transformed colonies are present at 10x, suggest that this concentration is not too high for transformants.
- **ATCC 53582 strain:**
  - Ampicillin: 0.2x and 0.5x had growth, 1x did not, thus use 1x concentration.
  - Kanamycin: 0.2x, 0.5x and 1x had growth, so must use a higher concentration (but test it first)
  - Chloramphenicol: even 1x had growth, so must grow at a higher concentration.

#### **Sequencing results of Biobricked pSEVA plasmids from 26<sup>th</sup> September:**

- MF311Ib (pSEVA311) not biobricked, original plasmid remained
- MF321b (pSEVA321) biobricked
- MF321c(pSEVA321) – sequencing reaction not successful
- MF331b (pSEVA331) – not biobricked, original plasmid
- MF331IIIa (pSEVA331) – Biobricked
- MF331IIIc (pSEVA331) - Biobricked

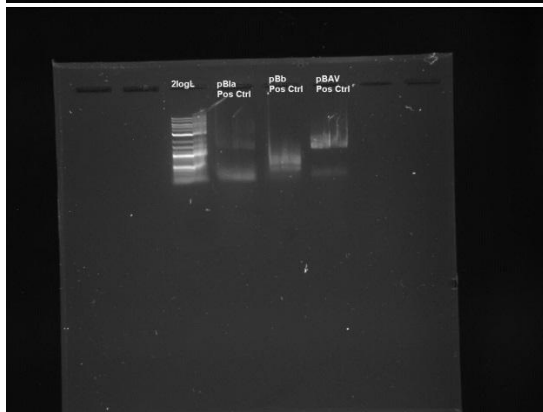
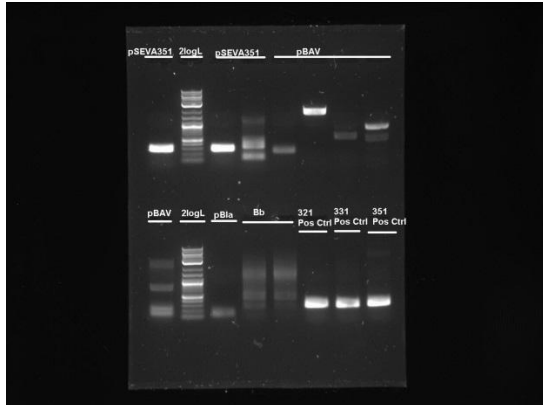
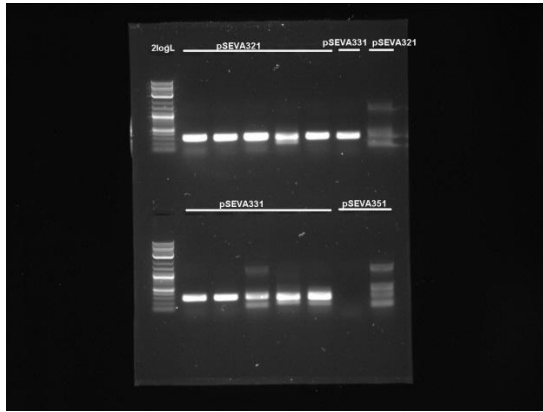
#### **Gel electrophoresis of diagnostic PCR of plasmids miniprepmed from KI and ATCC 53582 transformants**

- Expected band sizes:
  - pSEVA321 – 328bp
  - pSEVA331 -328bp
  - pSEVA351 - 328bp
  - pBla – about 700bp
  - pBAV - about 1000bp
- PCR with KI strains:



- PCR with ATCC 53582 strains:





- For both Kombucha-isolated and ATCC 53582 strain, pSEVA321, pSEVA331, pSEVA351, pBla replication is thus confirmed. pBAV replication is not yet, although it should be broad enough to replicate, and plates were not clear wrt colonies.

#### Diagnostic PCR of plasmids miniprepmed from ATCC 53582 for confirmation of plasmids in ATCC strain

- Master mix:

| Component                         | 25 µl Reaction |
|-----------------------------------|----------------|
| 5X Q5                             |                |
| Reaction Buffer                   | 5 µl           |
| 10 mM dNTPs                       | 0.5 µl         |
| 10 µM Forward Primer              | 1.25 µl        |
| 10 µM Reverse Primer              | 1.25 µl        |
| Template DNA                      | 1ul            |
| Q5 High-Fidelity DNA Polymerase   | 0.25 µl        |
| 5X Q5 High GC Enhancer (optional) | 5 µl           |
| Nuclease-Free Water               | 10.75          |

- Primers:
  - For pSEVA: iGEM 53, 54: Tm 61, 62
  - For pBAV: primer55-56 – Tm, 59, 61
  - For pBla: primer 63, 64) Tm, 60, 58
  - For Bb: iGEM7, iGEM8; Tm 61.5, 63

**26<sup>th</sup> September**

**Miniprepping transformed and 48H cultured ATCC 53582 cultures (transformed with pSEVA311-351, pBla, pBAV, and pSB1C3)**

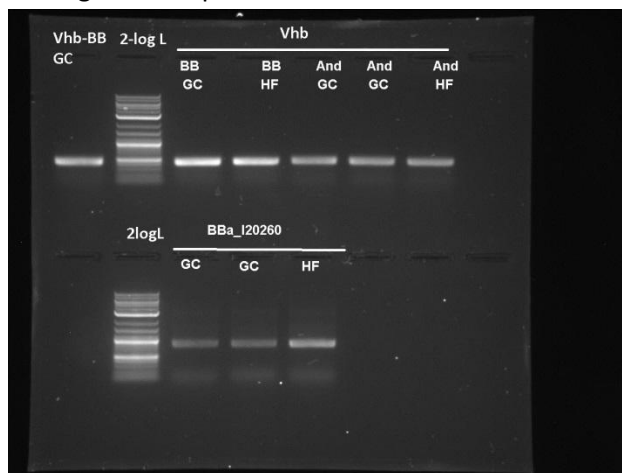
- Used Qiagen miniprep kit according to manufacturer's instructions

**Sequencing pSEVA plasmids converted into biobrick format, to confirm correct cloning**

- Used Source biosciences, according to their instructions

**Gel electrophoresis of PCR reactions done on 25<sup>th</sup> September**

- Sample number: 9+14=23 – 4 gels in total
- Adding Biobrick prefix and suffix to Vhb for Gibson assembly and part submission:



**Diagnostic PCR for confirmation of correct plasmids in KI transformants:**

- Plasmids: pSEVA 331, 351, 321 – iGEM 53, 54: Tm 61, 62
  - pBAV1K (primer55-56) – Tm, 59, 61
  - pBla-Vhb-122 (primer 63, 64) Tm, 60, 58

- Used Q5 polymerase for all, 50ul reaction, 1ul template DNA, according to manufacturer's instructions.

**25<sup>th</sup> September**

### **Miniprepping KI cultures transformed with various plasmids (inoculated on 15<sup>th</sup> Sept)**

- Used Qiagen Miniprep kit, according to manufacturer's instructions
- Miniprep yields low, most likely due to interfering cellulose and low starting cell content.

### **PCR reactions**

- **Amplifying out RBS-sfGFP-terminator construct for insertion behind arabinose inducible operon**
  - Forward primer: iGEM97(Tm 70, 52), reverse primer iGEM80 (tm 58)
  - DNA: XSM plasmid 13 (Bba\_I20260)
- **Putting Vhb coding sequence into BB format:**
  - Forward primer: iGEM102,(Tm 69,52) Reverse primer iGEM103(Tm 70, 49)
  - DNA:pBla-Vhb-122
- **Putting Vhb CDS behind Anderson promoters:**
  - Forward primer:iGEM100 (Tm:66, 52), reverse primer iGEM101 (Tm: 66, 49)
  - DNA: pBla-Vhb-122

PCR reaction conditions:

- Q5 polymerase, using touchdown 15 cycles, elongation 30 cycles, annealing temp. 52C
- Use both GC and HF buffer for both, 2x GC replicates for each
- **PCR extraction of BioBricks**
  - **BioBricks are the following:**
  - **K1033923**
  - **28**
  - **27**
  - **25**
  - **24**
  - **11,4**
  - **Forward primer iGEM79(56), iGEM80(58)**
  - **Use Q5 with both HF and GC buffer for each biobrick**

Q5 master mix same for both

- 55C

**22<sup>nd</sup> September**

### **Purifying *G.xylinus* ATCC 53582 and Kombucha-isolated strain gDNA using Zymo Clean and Concentrator kit 5**

- It seems there is a low level contamination present in gDNA

- Purified gDNA using Zymo kit according to manufacturer's instructions.
  - This could also not be contamination, but random fluctuations of 260/230 and 260/280 values due to low DNA concentration

#### **Miniprepping *G.xylinum* transformed with various broad-host range plasmids**

- 

#### **Miniprepping ligated and transformed pSEVA plasmids, that had Biobrick cloning site inserted into.**

**20<sup>th</sup> September**

#### **Digestion of Biobricks using XbaI, PstI**

- 20ul reaction volume, 0.5ul of each RE enzyme per reaction, using Cutsmart buffer
- Digested for 4hours at 37C, then placed at 4C fridge until further use

**19<sup>th</sup> September**

#### **gDNA extraction from pure inoculations of *G.xylinus* ATCC 53582 and Kombucha-isolated *G.xylinus***

- Used 1ml starting culture, which had been treated with 0.1% v/v cellulase at 30degC, 160rpm shaking overnight to degrade the cellulose pellicle.
- Used Qiagen Blood and Tissue kit for extraction, following the manufacturer's instructions.
  - Treated with proteinase K overnight at 56C.
  - Finished extraction in the morning
  - Measured DNA concentration using Nanodrop – some contamination present in all samples, resulting in low 260/230 ratios (high 230 peak)
- **Zymo purification of gDNA extractions**
  - Used Zymo DNA Clean and Concentrator 5 kit, following manufacturer's instructions
    - Eluted DNA in 60ul of distilled water –
    - Measured DNA concentration using Nanodrop – contamination amount increased manifold, with a very high 230nm peak. According to literature, this may be guanidine hydrochloride contamination. Cause of this unknown.

#### **Gel purification of digested BioBricks**

- Used Qiagen Minelute purification kit, following manufacturer's instructions

#### **Restriction digests of Biobricks for cloning into *G.xylinus*-compatible plasmids**

- Reaction set up:
  - 20ul reaction volume,
  - DNA for each BioBrick 15ul
  - Cutsmart buffer10x (diluted to 1x) 2ul

- 10 units of XpaI (20U/ul) 0.5ul
- 10 units of PstI (20U/ul) 0.5ul
- H2O 2ul
- Incubated at 37C for 2 hours, then heat inactivated at 80C for 30minutes.

#### Restriction digests of BioBricks for cloning into G.xylinus-compatible plasmids

- Reaction set up:
- 30ul reaction volume,
- 2ug of DNA for each BioBrick
- Cutsmart buffer10x (diluted to 1x) 3ul
- 10 units of XpaI (20U/ul) 0.5ul
- 10 units of SpeI (20U/ul) 0.5ul

#### 18<sup>th</sup> September

##### PCR of pBLA-Vhb-122 with primers iGEM85-86 and iGEM89-92 for insertion of BioBrick sites

- Used 50ul reaction volume:

| Component                  | 50 µl Reaction       | Final Concentration |
|----------------------------|----------------------|---------------------|
| Nuclease-free water        | 32.5 (31 with DMSOC) |                     |
| 5X Phusion HF or GC Buffer | 10 µl                | 1X                  |
| 10 mM dNTPs                | 1 µl                 | 200 µM              |
| 10 µM Forward Primer       | 2.5 µl               | 0.5 µM              |
| 10 µM Reverse Primer       | 2.5 µl               | 0.5 µM              |
| Template DNA               | 1ul                  | < 250 ng            |
| DMSO                       | (1.5 µl)             | 3%                  |
| Phusion DNA Polymerase     | 0.5 µl               | 1.0 units/50 µl PCR |

- Thermocycler conditions (touchdown PCR):
- 

#### 17<sup>th</sup> September

##### PCR of pSEVA311-351 with primers for insertion of BB prefix and suffix

- Used Phusion polymerase, 50ul reaction volume, master mix set up according to NEB instructions. Used 1ul of DNA for each reaction. For each DNA, used 2 replicates of HF buffer and 2 replicates of GC buffer.

#### **Plating out transformations of ATCC and KI strains made on 15<sup>th</sup> September**

- Plated out 300ul of each culture (900ul in total) to one of each plate with different antibiotic concentrations. Used 0.2x, 0.5x and 1x antibiotic concentrations for ATCC 53582 and 5x, 7x 10x for KI concentrations.

#### **16<sup>th</sup> September**

- Transformed ATCC 53582 strain in triplicates with each of pSEVA311-351, pBla-Vhb-122, pBAV1K, J23100 and pSB1C3. For each antibiotic concentration, added a negative control with no plasmid transformed. Electroporation conditions: 2.5kV, 25uF, time constant (actual) around 4-5ms. Cultured in 800ul HS+cellulase (0.1% v/v) media at 30C, 160rpm shaking. Added more cellulase (final concentration 0.2% v/v) to the medium 4 hours post growth, as some visible pellicles were present in the media. NB! Due to a potential fault in the shaker, shaking incubator may have been off for a period of 10-12 hours between 16<sup>th</sup> and 17<sup>th</sup> September.

#### **15<sup>th</sup> September**

#### **13<sup>th</sup> September**

- **Purification of PCR products containing ACS upstream, downstream and ACS ACBCD operon sequences**
  - Using Qiaquick PCR purification according to the manufacturer's protocols except:
    - Wait time for DNA elution 10 minutes
    - Elution in 50ul dH2O
- **Miniprepping pBla, pBAV, pSEVA and K510000, K510002**
  - Inoculated 20ml LB-antibiotic (respective to plasmid backbones) with single colonies from plates, cultured in 50ml Falcon tubes, 45deg angle, 200rpm, 37C shaking.
  -
- **Preparing electrocompetent cells in a shaking incubator**
  - Shaking at 160rpm, 30C, in 50ml Falcon tubes, 20ml media.
  - Added 0.1% v/v Celluclast cellulase into some cultures 4 cultures of ATCC without cellulase (0913MFIIa-d), 4cultures of ATCC with cellulase (0913MFIIIf-h), 4 cultures of KI-strain without cellulase (0913MFIVa-d) and with cellulase (0913MFIVf-h).
- **DNA sequencing of pSEVA311-351, pBla-Vhb-122 and pBAV1K**
  - pSEVA311, 321 failed – most likely due to low DNA concentration
  - pSEVA331 – sequencing confirms;
  - pSEVA341 – failed; peaks overlap with others continuously; could be contamination or too high concentration

- pSEVA351 – sequencing confirms
- pBAV1K-T5 – in general, the same as author's, but there is a deletion in the middle somewhere
- pBla- BLAST confirms *Vitreoscilla haemoglobin*; no alignment to the plasmid pBBR122 backbone itself
- **Gel electrophoresis of PCR products of ACS operon with primers iGEM05, iGEM06**
  - L-Quick-Load Purple 2-log DNA ladder, 5ul
  - DNA: ATCC 53582 3, 1ul
  - GC1 – PCR with 10ul GC enhancer, GC2 – PCR with 10ul GC enhancer, HF- PCR without 10ul GC enhancer



- Expected fragment size: 9066
- It seems GC2 was successful, whereas the others were not, even though GC1 was supposedly identical to GC2. – Set up things in duplicates/triplicates then, to account for any slight errors changing the conditions of the PCR

## 12<sup>th</sup> September

- **PCR of ACS operon using Q5 and primers iGEM05, iGEM06**

| Component                         | 50 µl          | Final Concentration |
|-----------------------------------|----------------|---------------------|
| 5X Q5 Reaction Buffer             | 10 µl          | 1X                  |
| 10 mM dNTPs                       | 1 µl           | 200 µM              |
| 10 µM Forward Primer              | 2.5 µl         | 0.5 µM              |
| 10 µM Reverse Primer              | 2.5 µl         | 0.5 µM              |
| Template DNA                      | 1 ul           | < 1,000 ng          |
| Q5 High-Fidelity DNA Polymerase   | 0.5 µl         | 0.02 U/µl           |
| 5X Q5 High GC Enhancer (optional) | (10 µ)         | (1X)                |
| Nuclease-Free Water               | 32.5 µl (22.5) |                     |

### Thermocycling Conditions:

98C, 30sec

—  
98C, 15sec

76C, 30sec; -1C per cycle  
72C, 9min  
x15

---

98C, 15sec  
61C, 30sec;  
72C, 9min  
x30

---

72C, 10min,  
4C hold

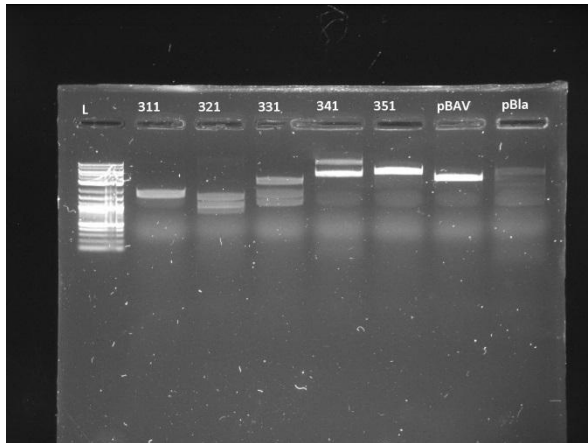
### **Touchdown**

- **Preparing plates and HS media for....**
- **Sequencing pSEVA plasmids and pBLA-Vhb-122 and pBAV1K-T5**
  - pSEVA311-pSEVA351: iGEM53 forward and iGEM 54 reverse primers
  - pBAV1K-T5: iGEM55, iGEM56 (forward and reverse)
  - pBla-Vhb-122: iGEM63, iGEM64 (forward and reverse)
  - Sequencing conditions:
  - Sequencing table labels:
    - MF1-pSEVA311
    - MF2-pSEVA321
    - MF3-pSEVA331
    - MF4-pSEVA341
    - MF5-pSEVA351
    - MF6-pBAV1k
    - MF7-pBla
    - MF11 to MF15- iGEM53
    - MF36 – iGEM55
    - MF57 –iGEM63
- **Transformation of ATCC 53582 and Kombucha-isolated G.xylinus strains using electroporation**
- **Miniprepping Biobricks**

### **11<sup>th</sup> September**

- PCR of upstream and downstream sequences worked, but not the whole operon. May need to redesign the primers.
- PCR of pSEVA plasmids with sequencing primers to verify their nature and construct.
- **Restriction digest of pSEVA311-351, pBAV1K-T5 and pBla-Vhb-122 for analysis**
  - pSEVA311-351 using NgoMIV o
  - pBAV1K-T5 using EcoRI
  - pBla-Vhb-122 using NotI restriction digests.





- Gel: 90V, 45min, 3ul SYBR in 50ml 1% agarose gel. 5ul NEB Quickload 2-log ladder
- Transformation of pBla-Vhb-122 into Kombucha-isolated strain and ATCC 53582 strain
- Analysis of extracted gDNA from Kombucha-isolated strain and ATCC 53582 strain

10<sup>th</sup> September

Genomic DNA extraction from ATCC 53582 and Kombucha-isolated *G.xylinus* strains using Qiagen Blood and Tissue kit

- Eluted twice with 200ul PCR grade H2O, into different 1.5ml tubes

PCR of pSEVA plasmids with primers iGEM57 – iGEM60 for insertion of BioBrick RE sites

- Set up for reactions (also for each primer pair, a negative control without DNA was added:

| DNA      | Primers                          |
|----------|----------------------------------|
| pSEVA311 | iGEM57-iGEM58                    |
| pSEVA311 | iGEM59-iGEM60                    |
| pSEVA321 | iGEM57-iGEM58                    |
| pSEVA321 | iGEM59-iGEM60                    |
| pSEVA331 | iGEM57-iGEM58                    |
| pSEVA331 | iGEM59-iGEM60                    |
| pSEVA341 | iGEM57-iGEM58                    |
| pSEVA341 | iGEM59-iGEM60                    |
| pSEVA351 | iGEM57-iGEM58                    |
| pSEVA351 | iGEM59-iGEM60                    |
| No       | iGEM 57-58; iGEM59-60 separately |

- 50ul reaction volume. Master mix component below:

| Component | 50 µl Reaction | Final Concentration |
|-----------|----------------|---------------------|
|-----------|----------------|---------------------|

|                            |          |                     |
|----------------------------|----------|---------------------|
| Nuclease-free water        | 31 (GC)  |                     |
| 5X Phusion HF or GC Buffer | 10 µl    | 1X                  |
| 10 mM dNTPs                | 1 µl     | 200 µM              |
| 10 µM Forward Primer       | 2.5 µl   | 0.5 µM              |
| 10 µM Reverse Primer       | 2.5 µl   | 0.5 µM              |
| Template DNA               | 1ul      | < 250 ng            |
| DMSO                       | (1.5 µl) | 3%                  |
| Phusion DNA Polymerase     | 0.5 µl   | 1.0 units/50 µl PCR |

- Thermocycler conditions (touchdown PCR):

98C, 30 sec;

98C, 15 sec

68C, 30sec, -1C decrease per cycle

72C 4 minutes

X15

98C, 15 sec

53C, 30sec

72C, 4 minutes

X 25

72C ,10 minutes

4C, hold

**PCR of ACS synthase operon from *E.coli* chromosome, as well as upstream and downstream genes using primers iGEM01-iGEM06**

**Sample set up:**

**PCR master mix:**

| Component                  | 50 µl Reaction     | Final Concentration |
|----------------------------|--------------------|---------------------|
| Nuclease-free water        | 31.5 (HF), 30 (GC) |                     |
| 5X Phusion HF or GC Buffer | 10 µl              | 1X                  |

|                        |          |                     |
|------------------------|----------|---------------------|
| 10 mM dNTPs            | 1 µl     | 200 µM              |
| 10 µM Forward Primer   | 2.5 µl   | 0.5 µM              |
| 10 µM Reverse Primer   | 2.5 µl   | 0.5 µM              |
| Template DNA           | 2ul      | < 250 ng            |
| DMSO (with GC buffer)  | (1.5 µl) | 3%                  |
| Phusion DNA Polymerase | 0.5 µl   | 1.0 units/50 µl PCR |

- Thermocycler conditions (touchdown PCR):

98C, 30 sec;

98C, 15 sec

75C, 30sec, -1C decrease per cycle

72C , 8 minutes

X15

98C, 15 sec

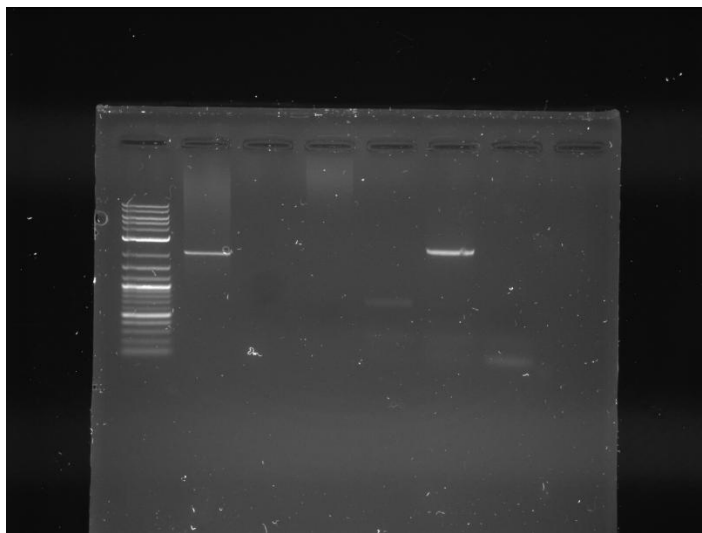
61C, 30 sec

72C, 8 minutes

X 25

72C ,10 minutes

4C, hold



- 
- Legend: 1-Ladder; 2 –primers iGEM1,2 in CG+DMSO, 3-iGEM1,2 in HF buffer, 4-

9<sup>th</sup> September

Extracting gDNA from ATCC 53582 and Kombucha-isolated strain

- On 8<sup>th</sup> September, added 0.1% (v/v) cellulase from T.reesei to each culture; incubated at room temp. without shaking for 6 hours – cellulose pellicles were not fully degraded as a result. Then transferred the cultures to 30C without shaking for 2 hours – even then cellulose was not fully degraded. Placed cultures to 4C overnight to stop growth.
- On 9<sup>th</sup> September, added further cellulose (0.2% v/v final concentration) to cultures under flow hood, using sterile technique throughout; incubated with shaking at 37C for 2 hours (shaking VWR standard analogue shaker, at speed 4)

#### **Preparing electrocompetent ATCC 53582 and Kombucha-isolated strain cells**

- Added T.reesei cellulose (from Sigma-Aldrich) to 0.2% v/v final concentration under fume hood to cultures grown for electrocompetent cell preparation. Cultures were grown standing at 30C for 3-5 days (see details from online lab book). Incubated cultures shaking at 37C for 6 hours (VWR shaker, speed 4). Samples then stored at 4C until further manipulation.

#### **Different feedstocks experiment**

- ATCC 53582: Growth in all cultures except HS-acetate and negative controls
- KI-strain Growth in all cultures except HS-acetate, HS-sucrose and negative controls
- **Terminated the experiment by placing the cultures at 4C.**

#### **Natural antibiotics resistance experiment using plate**

- **ATCC 53582:** None in neg, control. Many in 2/2 positive controls. None in any antibiotic-containing plates
- **Kombucha-isolated** strain: No growth in neg, 2/2 in positive. Cam: 3/3 in 1x, no growth in others. Kan: 3/3 in 1x, 3/3 (fewer, in the edges) in 4x, none in 16x. Amp: 3/3 in 1x, 3/3 in 4x (fewer), none in 16x.
- **Terminated the experiment by placing the cultures at 4C.**

#### **Natural antibiotics resistance experiment using liquid HS**

- **ATCC 53582:** No growth in any cultures, including positive controls. Need to repeat this experiment
- **KI-strain:** Growth in 2/3 positive; no growth in negative. HS-chloramphenicol: 3/3 in 1x, no growth in others. Amp: very thin, barely visible pellicle in 1/3 1x, 1 strong in 1/3 4x, none or 1 very small in 1/3 16x. Kan: 1 large in 1/3 1x, none in 4x, 16x
- **Terminated the experiment by placing the cultures in 4C.**

5<sup>th</sup> September

Used Phusion polymerase, master mix below:

|                |       |     |     |     |
|----------------|-------|-----|-----|-----|
| H2O            |       | 390 | 390 | 260 |
| Phusion buffer | 10ul  | 120 | 120 | 80  |
| 10mM dNTPS     | 1ul   | 12  | 12  | 8   |
| Phusion        | 0.5ul | 6   | 6   | 4   |

|                            |              |           |           |           |
|----------------------------|--------------|-----------|-----------|-----------|
| <b>polymerase</b>          |              |           |           |           |
| <b>10uM Forward primer</b> | <b>2.5ul</b> | <b>30</b> | <b>30</b> | <b>20</b> |
| <b>10um Reverse primer</b> | <b>2.5ul</b> | <b>30</b> | <b>30</b> | <b>20</b> |
| <b>Template DNA</b>        | <b>1ul</b>   | <b>1</b>  | <b>1</b>  | <b>1</b>  |
|                            |              |           |           |           |
|                            |              |           |           |           |

**Thermocycler protocol:**

94C,3 min  
94C, 30sec  
69C, 30sec; -1C per cycle  
72C, 5 min  
X10  
94C, 30sec  
59C, 30sec  
72C, 5 min  
X25  
4C hold

| <b>Component</b>           | <b>20 µl Reaction</b> | <b>50 µl Reaction</b> | <b>Final Concentration</b> |
|----------------------------|-----------------------|-----------------------|----------------------------|
| Nuclease-free water        | to 20 µl              | to 50 µl              |                            |
| 5X Phusion HF or GC Buffer | 4 µl                  | 10 µl                 | 1X                         |
| 10 mM dNTPs                | 0.4 µl                | 1 µl                  | 200 µM                     |
| 10 µM Forward Primer       | 1 µl                  | 2.5 µl                | 0.5 µM                     |
| 10 µM Reverse Primer       | 1 µl                  | 2.5 µl                | 0.5 µM                     |
| Template DNA               | variable              | variable              | < 250 ng                   |
| DMSO (optional)            | (0.6 µl)              | (1.5 µl)              | 3%                         |
| Phusion DNA Polymerase     | 0.2 µl                | 0.5 µl                | 1.0 units/50 µl PCR        |

**29<sup>th</sup> August**

-Received the *G.xylinus* ATCC53582 strain from USA. Stored the strain in a parafilm double sealed secondary container at 4C.

**27<sup>th</sup> August**

**MF**

**Miniprepping BioBricks transformed yesterday** – all cultures contained bacteria, however growth room temperature was around 38-39C. Miniprep DNA using Qiagen Miniprep kit, following manufacturers with the exception of dissolving DNA in EB buffer for 10 minutes before centrifugation. Then measured DNA concentrations using NanoDrop. Stored samples in -20C freezer.

**26<sup>th</sup> August**

**MF**

**Transforming BioBricks into *E.coli*** – Transformed the following plasmids into *E.coli*:

- 1) plasmids containing BioBricks Bba\_C0012 and Bba\_I13600
  - 2) SEVA plasmids pSEVA pSEVA311, pSEVA321, pSEVA331, pSEVA341, pSEVA351
- Used chemical transformation protocol described at XXXXX.

***E.coli* and *G.xylinus* co-culture experiment**-Prepared and autoclaved HS-glucose, HS-glycerol and HS-glucose-glycerol media. All samples were set up in triplicates.

**23<sup>rd</sup> August**

**MF**

**Making LB-agar plates with different, higher antibiotic concentrations to determine LD<sub>50</sub> of ampicillin, kanamycin and chloramphenicol of Kombucha-isolated Acetobacter strain-**

**Preparing chemically competent *E.coli* DH10B cells** – Used the protocol described in XXX (also other strains if necessary)

**Miniprepping 20 Biobricks inoculated on the 22<sup>nd</sup> August-**

**Transforming *E.coli* DH10B cells with plasmid pBla-Vhb-122 received from Dr Lee-**

**Measuring cellulose production of Kombucha-isolated Acetobacter grown on different feedstocks-**

**Corresponding with Dr Lee to confirm receipt of the plasmid and measuring DNA concentrations using NanoDrop-**

**Ordering primers for pBla-Vhb-122 sequencing –**

**Designing primers for Biobricks and pBAV1K-T5-gfp modification -**

**22<sup>nd</sup> August**

**MF**

**Transforming Biobricks into *E.coli* DH10B** – Growth was seen in all plates except for the one containing Bba\_XXXX transformed *E.coli*. Inoculated 2 colonies from each plate into 15ml Falcon

tubes containing 5ml LB-antibiotic (40-2=38 in total). Incubated at 37°C shaking (45° angle) overnight. NB! Growth room temperature may vary – recently temperatures between 35 and 39 have been reported.

**Designing primers for pBla-Vhb-122 sequencing** – There is not full sequence available in any checked databases (GenBank, company websites). Designed primers based on *bla* promoter sequence to determine the layout of the plasmid so far.

**Laboratory maintenance** – Prepared LB medium, cleared up lab, washed glassware.

**21<sup>st</sup> August**

**MF**

**Transforming Biobricks into E.coli DH10B** - 18 Biobricks in pSB1C3 (CamR) and 2 Biobricks in pSB1A2(AmpR) – requires 18 plates. 20 15ml tubes and 20 1.5ml microcentrifuge tubes. Prepared all of the necessary tubes and plates, labelled accordingly. Transformed the Biobricks in the evening, after returning from Thames Water purification plant. Used a chemical transformation protocol ( see PROTOCOL) for transformation. Transformed 20 BioBricks in total, using plates with respective antibiotics and E.coli DH10B cells. Incubated at 37C standing.

**20<sup>th</sup> August**

**MF**

**Testing natural antibiotic resistance of Kombucha-isolated strain** – Strain grown since 14<sup>th</sup> August at 30C, 60rpm shaking. All cultures except one replicate of HS-Ampicillin contain visible amounts of cellulose, indicating that this strain is naturally resistant to all tested antibiotics (ampicillin, kanamycin, chloramphenicol) at the concentrations tested. Crap.

**Testing growth of Kombucha-isolated G.xylinus strain on different feedstocks –**

**18<sup>th</sup> August**

**MF**

**Testing ATCC23769 for contamination** – Plates grown for 48h at 30C on HS-agar displayed thick growth of colonies with E.coli – discarded the plates. Furthermore, liquid HS-media inoculated with supposed ATCC23769 displayed no cellulose production and were cloudy, indicating *E.coli* presence. Most likely, the strain sent from U.Röslig is not Acetobacter, but E.coli

**Testing natural antibiotic resistance of Kombucha-isolated and U.Röslig's strain.** – Small HS media inoculated with Kombucha-isolated strain displayed small pellicles in cultures containing 1) no antibiotics, 2) kanamycin, 3) chloramphenicol, but no pellicle in ampicillin-containing media. HS media inoculated with U.Röslig's strain displayed no pellicles in any culture, however cultures with 1) no antibiotics and 2) kanamycin were cloudy. This again points to E.coli (namely KanR one), however I will allow one more day of growth before making the final conclusion.

**16<sup>th</sup> August**

**Growing up and testing pure ATCC23769 strain sent from U.Röslig lab.**

Prepared new HS media to remove possibility of contamination due to contaminated media.

**Testing ATCC23769 for contamination**

Plates grown overnight were filled with small colonies (Acetobacter colonies are expected to be visible within 48h, not overnight), with *E.coli* colour and morphology (as viewed under microscope with 50x magnification). Furthermore, plates smelled like *E.coli*, all of which (too fast growth rate, colony morphology and cloudy liquid cultures) points to *E.coli* contamination. Thus, PCR done yesterday (with supposed ATCC23769 DNA) failed due to this reason. I have discarded plates and cultures inoculated with contaminated pre-culture.

**Making glycerol stocks of Kombucha-isolated Acetobacter strain**

Used 500ul of Kombucha-isolated Acetobacter culture, added 500ul of 50% glycerol. Stored in -80C.

**15<sup>th</sup> August**

**MF**

**Testing ATCC23769 for contamination**

ATCC23769 cultures grown for 48+ hours on HS media were cloudy, which is unlike Acetobacter. To confirm purity, plated out 3 HS-agar plates with Acetobacter, as well as 3 liquid HS cultures (15ml in 50ml falcon tubes) and incubated standing at 30C.

**PCR of bcsABCD operon from G.xylinus genomic DNA**

- PCR master mix:

|                          |       |  |
|--------------------------|-------|--|
| HF buffer                | 140ul |  |
| dNTPs                    | 14ul  |  |
| Forward primer           | 35ul  |  |
| Reverse primer           | 35ul  |  |
| Template DNA (26.6ng/ul) | 42ul  |  |
| DMSO                     | 21ul  |  |
| Phusion polymerase       | 7ul   |  |
| H2O                      | 396ul |  |

- Master mix then divided into 2 equal volumes, 21ul of template DNA 1 and 2 then added to each respectively. These then divided into 14, 50ul aliquots which were used for the PCR.
- Thermocyclin conditions:
- 98C 30s
- 57C 30s (temp gradient from 52 to 62C)
- 72C for 12 minutes
- X35



### **Seeding cultures for different substrates experiment:**

Measured OD600 of Kombucha-isolated and ATCC23769 Acetobacter strain pre-cultures (seed cultures) using a Nanodrop. Based on OD600, adjusted volume of inoculum to add equal amount of cells into each culture.

Set up 500ml conical flasks containing 50ml of medium. Both Kombucha-isolated and ATCC 23769 strains were tested on 5 different media (HS-glucose, HS-sucrose, HS-acetate, HS-glycerol, Kombucha tea medium) in triplicates. Flasks were set to incubate without shaking on 30C.

### **Creating a pre-culture of *G.xylinus***

Added 30ul of Celluclast cellulase into 15ml of 48+h grown cultures (0.2% v/v). Cultured with shaking for 2 hours at 30C, 60rpm, in an upright position.

### **Extracting parts BBa\_J23100, BBa\_J23070, BBa\_J23118 from overnight BL21(DE3) cultures**

Used Qiagen Miniprep kits starting 10ml of culture (otherwise following manufacturer's instructions). Quantified minipreped plasmid DNA concentration using Nanodrop – minipreps were successful, yielding a high amount of DNA (>100ng/ul).

### **Plasmid BL21(DE3)**

-Found E.coli overnight cultures in the shaker out of place and ruined; most likely de-attached during cultivation. Three cultures (luckily one from each plate) were intact – will use them for minipreps. Have decontaminated the shaker and everything else that came into contact with the culture using Virkon.

**14<sup>th</sup> August**

**MF**

**Transforming BL21(DE3) with Biobricks** - Transformants from yesterday growing – colonies present on samples 0813MFVI – 0813MFVIII.

Inoculated two colonies from each plate into 20ml LB+amp medium (in 50ml Falcons). Grew with shaking at 37C, 140rpm (due to problems with higher shaking speed).

### **Culturing *G.xylinus* strains on different media**

Set up 15 and autoclaved 15 additional flasks for ATCC23769 strain. Waiting for seed culture to grow in order to begin the experiment.

### **Testing antibiotic resistance of *G.xylinus* strains**

Set up additional HS-antibiotic containing Falcon tubes (50ml tubes containing 15ml of HS+ ampicillin, chloramphenicol or kanamycin). Waiting seed culture to grow to begin the experiment.

**13<sup>th</sup> August**

**MF**

- **Transforming *E.coli* BL21(DE3) with biobricks.**
  - Followed chemical transformation protocol from XSM. NB! Water bath temp. might not have been fully at 42C, but lower. This could cause massively decreased transformation

efficiencies. NB! It seems the thermometer was broken, so real temp. Plated out on LB-agar with ampicillin, left to grow at 37C.

- **Culturing Kombucha-isolated *G.xylinus* on different media**
  - Set up 15 shaker flasks, autoclaved them. Measured OD<sub>600</sub> of Kombucha-isolated *G.xylinus* using Nanodrop – concentration below detection limit, so requires further culturing.
- **Preparing electrocompetent *G.xylinus* cells**
  - Preparing HS-cellulase media. Will use four 50ml Falcon tubes (15mL media in each) when seed culture concentrated enough to be used.
- **Testing natural antibiotic resistance of Kombucha isolated *Acetobacter***
  - Add 15ml HS medium to 4x3=12 50ml Falcon tubes with antibiotics as following: Amp, Kan, Chloramphenicol, no antibiotics. Will continue when seed culture ready.
- **Making glycerol stocks of Kombucha-isolated *G.xylinus***
  - Can use seed culture (after digestion with cellulase) when it is ready.
- **Gluconacetobacter 3D growth**

DSC

**28<sup>th</sup> July**

#### **MF - Microscopy of *G.xylinus* colonies**

Observed plates of 0726MFI – III under low magnification (50x); no contamination was detected in any of the plates. Colony morphology roughly similar, although 0726MFI colonies appeared often larger than 0726MFII (this could be attributed to small differences in e.g. temperature or aeration). 100ul is too much, as colony density was high – for obtaining single colonies, 10-20ul should suffice.

#### **MF – Inoculating *G.xylinus* cultures from single colonies**

A single colony was marked on each of 0726MFI – III plates and used to inoculate 15ml of *Gluconacetobacter* medium (in a 50ml tube). Cultures were placed on 30C, no shaking for growth. Sterile technique was used throughout.

**26<sup>th</sup> July**

#### **MF - Aliquoting *G. xylinum* cultures**

Made 1ml aliquots of cultures 0721MFI (aliquot named 0726MFI), 0721MFIV (aliquot named 0726MFII) and 0722MFI (aliquot named 0726MFIII).

Plated out 100ul of 0721MFI, 0721MFIV and 0722MFI onto *Gluconacetobacter*-agar media.

Incubated at 30C.

After plating, discarded all 0721MF and 0722MF cultures.

**25<sup>th</sup> July**

#### **MF - Monitoring growth and microscopy**

Readings: temp of samples 0721MFI to 0721MFVIII and 0722MFI grown in 30C, not shaking. A thin sheet visible in the flasks of 0721MFI to IV and 0722MFI (inoculated from small colonies). Prepared microscopy slides of all cultures. Microscopy suggested that there are no contaminants in cultures

0721MFI, IV and 0722MFII, thus we have likely isolated a strain of cellulose producing *G.xylinus* or another species from Kombucha culture.

## **24<sup>th</sup> July**

### **MF - Monitoring growth and microscopy**

Checked growth of colonies from re-streaked plates and culture inoculated with *G.xylinus* on 22<sup>nd</sup> July. Colonies on re-streaked plates all small, same morphology as previously observed for bacterial colonies – most likely *G.xylinus*. Prepared 3 slides from culture – very low number of cells present, hint of cellulose fibrils - very few seen under microscope, could be contamination/clumping. Low growth possibly due to improperly prepared media (glucose and amino acids autoclaved together, which may result in toxic compounds due to Maillard reaction).